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Drosophila cohesins DSA1 and Drad21 persist and colocalize along the centromeric heterochromatin during mitosis

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Abstract

Sister chromatid cohesion in eukaryotes is maintained mainly by a conserved multiprotein complex termed cohesin. Drad21 and DSA1 are the *Drosophila* homologues of the yeast Scc1 and Scc3 cohesin subunits, respectively. We recently identified a *Drosophila* mitotic cohesin complex composed of Drad21/DSA1/DSMC1/DSMC3. Here we study the contribution of this complex to sister chromatid cohesion using immunofluorescence microscopy to analyze cell cycle chromosomal localization of DSA1 and Drad21 in S2 cells. We observed that DSA1 and Drad21 colocalize during all cell cycle stages in cultured cells. Both proteins remain in the centromere until metaphase, colocalizing at the centromere pairing domain that extends along the entire heterochromatin; the centromeric cohesion protein MEI-S332 is nonetheless reported in a distinct centromere domain. These results provide strong evidence that DSA1 and Drad21 are partners in a cohesin complex involved in the maintenance of sister chromatid arm and centromeric cohesion during mitosis in *Drosophila*.

Keywords: Cohesin; Chromosome segregation; Sister chromatid cohesion; Centromere; *Drosophila*; Mitosis

1. Introduction

Chromosome segregation during mitosis is potentially the most dangerous process in the life of a cell. Errors in this process induce chromosome instability, which is associated with many cancers and is the cause of several birth defects in man. DNA replication produces two identical copies termed sister chromatids, which are physically linked until the metaphase/anaphase transition. Sister chromatid cohesion is maintained by a multiprotein complex called cohesin. This complex, which was first characterized in *Saccharomyces cerevisiae*, is composed of four subunits: Smc1 and Smc3, which belong to the structural maintenance of chromosomes (SMC) family of proteins, and the two non-SMC components, Scc1 and Scc3 (reviewed in Haering and Nasmyth, 2003). A similar complex has been identified in *Schizosaccharomyces pombe, Xenopus* and man (Tomonaga *et al.*, 2000; Losada *et al.*, 2000; Sumara *et al.*, 2000).

In budding yeast, the cohesin complex dissociates from chromatin at the metaphase/anaphase transition, allowing chromosome segregation, and the Scc1/Rad21 subunit is the principal substrate of separase proteolytic cleavage (Uhlmann *et al.*, 1999). In vertebrates, however, release of sister chromatid cohesion is a two-step process. The bulk of cohesin complexes, located mainly at chromosome arms, is released from chromatin during prometaphase by a non-proteolytic mechanism, although a pool of centromere-associated cohesin persists. Dissociation of cohesin from chromosome arms depends on the activity of Polo-like (Sumara *et al.*, 2002) and Aurora B (Losada *et al.*, 2002) kinases, but is independent of the separase pathway, whereas centromeric cohesion, which is maintained until the metaphase/anaphase transition, is lost *via* a mechanism that involves the Scc1 cleavage by separase (Waizenegger *et al.*, 2000). Difficulties have nonetheless been reported in detecting cohesin by immunofluorescence in vertebrate metaphase chromosomes. In meiosis, separase is also necessary to cleave the Scc1 meiotic homolog and to dissociate cohesin

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from chromosome arms in yeast (Buonomo *et al.*, 2000), worm (Siomos *et al.*, 2001) and mammals (Herbert *et al.*, 2003), but not in *Xenopus* (Peter *et al.*, 2001; Taieb *et al.*, 2001). The apparent protection of cohesin in the vicinity of centromeres is intriguing, as it could be conferred by distinct subunit composition of arm and centromeric cohesin complexes, and/or by interaction of the cohesin complex with other centromeric proteins. Analysis of cohesin complex composition in different organisms and its involvement in sister chromatid arm and centromeric cohesion will thus help elucidate this aspect of chromosome segregation.

In searching the *Drosophila* genome, we found two sequences for the putative homologues of mammalian Scc3/SA/STAG proteins. One corresponds to that known as DSA (now DSA1), and the second is a sequence (accession #AAF47494) that we term DSA2. Biochemical and RNAi experiments in *Drosophila* S2 cells suggest that Drad21 and DSA1 are subunits of the same cohesin complex (Vass *et al.*, 2003), but to our knowledge no cytological results have yet been reported supporting this hypothesis. It is thus not known whether DSA1 localizes in chromatid arms and/or in the centromere during *Drosophila* mitosis. Here we analyze the cytological expression of DSA1 and compare it to that of Drad21 throughout the cell cycle in S2 cells. In addition, we studied the colocalization of DSA1 and MEI-S332, a *Drosophila* protein required for centromere cohesion during mitotic and meiotic divisions (Kerrebrock *et al.*, 1995).

2. Results and discussion

2.1. DSA1 expression pattern during the S2 cell cycle

We analyzed the cell cycle distribution of DSA1 by immunofluorescence (Fig. 1). In interphase cells, DSA1 was present in the nucleus with the exception of the nucleolus, and the cytoplasm showed diffuse staining (Fig. 1A). In mitotic prophase, DSA1 labeling was distributed along the condensing chromosomes (Fig. 1B). We detected foci of DSA1 signals located at heterochromatic centromeric regions (Fig. 1B), which were more evident when chromosomes condensed later in prometaphase. In this phase, DSA1 showed discrete localization along the condensed chromosomes, with intense labeling in the centromeric regions (Fig. 1C), and a fainter one along and between sister chromatid arms (Fig. 1C, arrowheads). When chromosomes were highly condensed in metaphase, we detected an intense DSA1 signal at the centromeric regions across the center of the cell. We also observed labeling at the spindle poles, and diffuse staining of the spindle (Fig. 1D). After chromatid separation in anaphase, the spindle poles were the most intensely labeled structures, whereas no DSA1 signal was detected in chromosomes (Fig. 1E). Telophase cells were characterized by DSA1 labeling in chromatin (Fig. 1F). Our results provide evidence that, as in vertebrate cultured cells, DSA1 cohesin disappears from the chromatid arms during

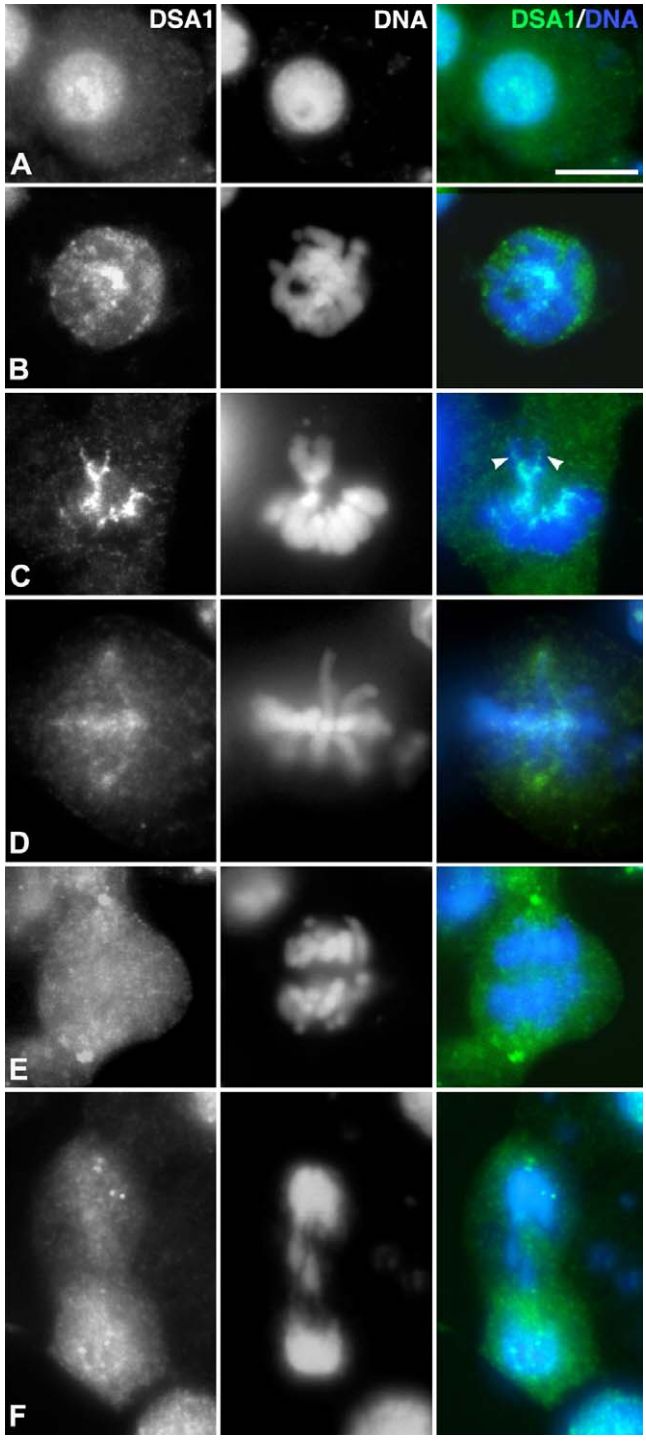

Fig. 1. **DSA1 immunolocalization during the S2 cell cycle**. (A) Interphase showing strong nuclear staining. (B) Prophase showing DSA1 restriction to condensing chromosome regions. (C) Prometaphase in which condensed chromosomes show strong DSA1 staining at centromeres and faint labeling between sister chromatid arms (arrowheads). (D) Metaphase. DSA1 is highly restricted at centromeres. Note staining of cell poles as well as diffuse labeling decorating spindle microtubules. (E) Anaphase. Chromatin-associated DSA1 is undetectable, but the cell pole signal persists. (F) Telophase cell showing faint DSA1 chromosome staining. In merge images, DSA1 staining appears in green and DAPI-stained DNA in blue. Scale bar = 5 μm.

the prometaphase/metaphase transition, whereas centromeric DSA1 persists until sister chromatid separation at anaphase onset.

2.2. DSA1 and MEI-S332 are found in distinct centromere domains

For a more detailed analysis of DSA1 in metaphase chromosomes, we colocalized DSA1 with various centromeric proteins (Fig. 2). In *Drosophila* cells, the MPM2 antibody recognizes mitotic phosphoepitopes located predominantly in the kinetochores (Logarinho & Sunkel, 1998). In S2 metaphase chromosomes, DSA1 appeared as bright bands parallel to the equatorial plate. These bands were restricted to the centromeric heterochromatin, as revealed with DAPI (Fig. 2A). In these cells, the MPM2 antibody labeled pairs of dots at centromeres; each dot faced a different cell pole and represented a sister kinetochore (Fig. 2A). At each centromere, DSA1 was distributed along the junction of paired sister chromatids and perpendicular to the hypothetical axis connecting the MPM2-labeled kinetochores (Fig. 2B). After comparing the labeling with the DAPI image, it was evident that DSA1 was not only restricted to the pairing domain beneath kinetochores, but also extended along the length of the centromeric heterochromatin (Fig. 2B).

The *Drosophila* MEI-S332 protein localizes to centromeres in mitosis and meiosis, and is required for the maintenance of centromere cohesion in meiosis II (Kerrebrock *et al.*, 1995; Moore *et al;*, 1998). Since in mitotic chromosomes this protein appears at the centromere pairing domain (Lopez *et al.*, 2000), as does DSA1, we double immunolabeled the two proteins to test for colocalization (Fig. 2C). MEI-S332 yielded a typical signal of two dots joined by a connecting strand, whereas DSA1 appeared as bright signals perpendicular to MEI-S332 staining. These two proteins thus occupy different centromere domains. This is noteworthy, since if MEI-S332 also maintains centromere cohesion in mitotic chromosomes, it would not act directly on the entire cohesin subunit DSA1 in heterochromatin, but we cannot discard the possibility that MEI-S332 interacts

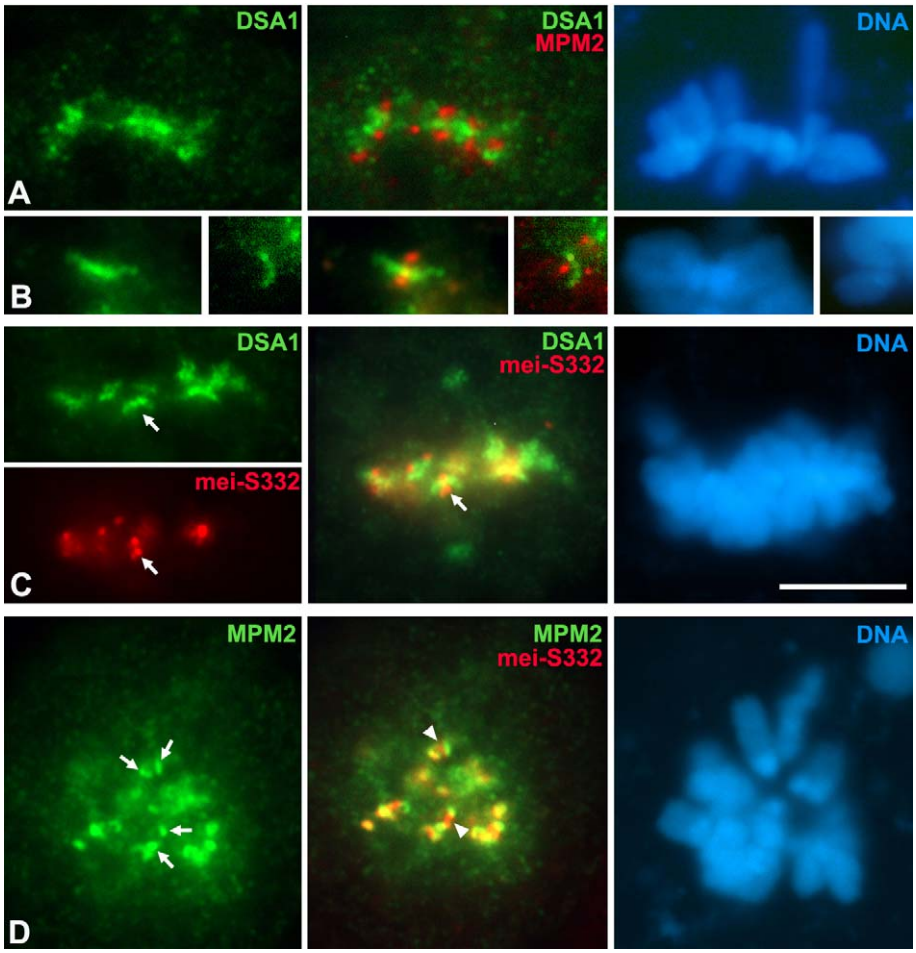

Fig. 2. **Immunolocalization of centromere proteins on S2 metaphase chromosomes**. Double immunolabeling of DSA1 (green) with MPM2 (red) or MEI-S332 (red) and DAPI (blue) counterstaining at metaphase. (A) A bright DSA1 signal is located at centromere regions where pairs of MPM2 dots along the spindle equator denote sister kinetochores. (B) Two selected metacentric chromosomes show that DSA1 is not restricted to the kinetochore pairing domain, but is also observed along the length of centromeric heterochromatin. (C) A single centromere is arrowed in the same focal plane of a metaphase cell. The same centromere is indicated in the merged figure (arrow). The signals of both antibodies are perpendicular to each other and colocalize only at the pairing domain. (D) MEI-S332 (red, arrowheads) appears as a continuous signal between MPM2-labeled kinetochores (green, arrows). Note that the two antibodies showed a significant degree of overlap. Scale bar = 5 μm.

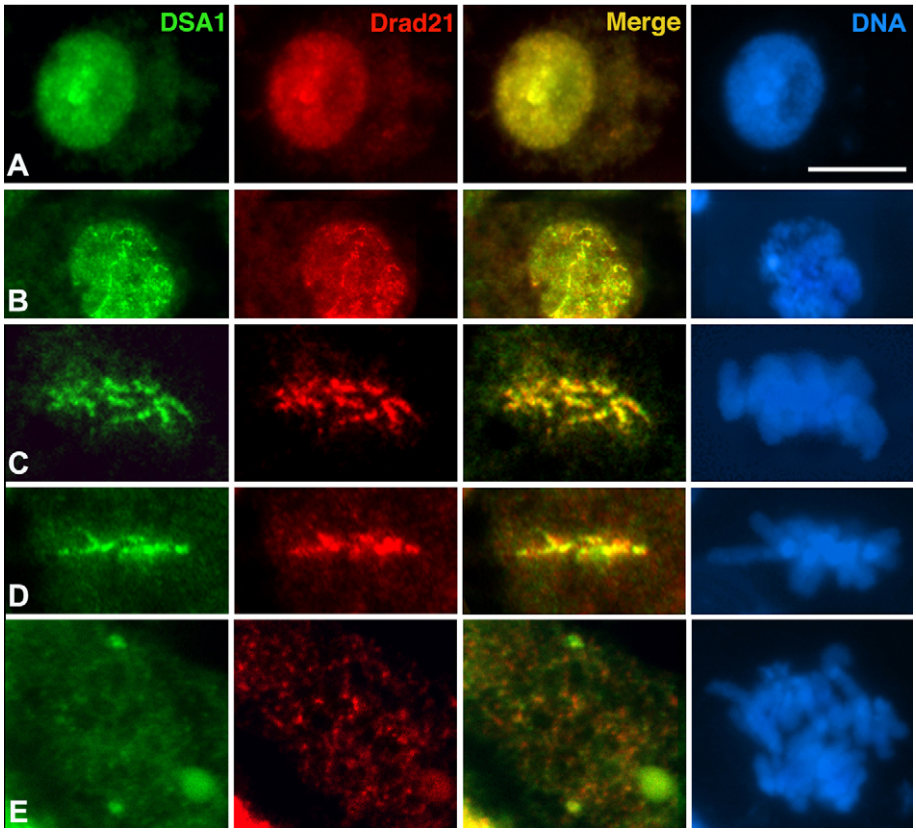

Fig. 3. **Colocalization of DSA1 and Drad21 cohesin subunits during mitosis in S2 cells**. Double immunolabeling of DSA1 (green) and Drad21 (red); regions of colocalization are yellow. DNA is stained with DAPI (blue). Interphase (A), prophase (B) prometaphase, (C) metaphase (D) and anaphase (E). DSA1 and Drad21 colocalize completely throughout the cell cycle, except in anaphase where the spindle poles only show DSA1 staining. Scale bar = 5 μm.

with colocalizing DSA1. Based on sequence homology, it was recently reported that MEI-S332 is the *Drosophila* homologue for the centromeric cohesion protector shugoshin from yeast (Kitajima *et al.*, 2004; Rabitsch *et al.*, 2004; Marston *et al.*, 2004). Our results on localization of this protein concur with this finding.

The MEI-S332 labeling observed is similar to that described by Blower & Karpen (2001), who reported that MEI-S332 was slightly displaced from the kinetochores. We colocalized MEI-S332- and MPM-2-labeled kinetochores to analyze MEI-S332 distribution. We observed MEI-S332 between sister kinetochores, and that it colocalized partially with them (Fig. 2D). It was proposed that MEI-S332 recruitment to centromeres is dependent on functional centromeric chromatin, determined by the presence of the inner kinetochore protein CID (Blower & Karpen, 2001); the partial colocalization of MEI-S332 and kinetochores detected here thus supports that assumption.

Centromeres are heterochromatic in many organisms, and functional links between heterochromatin and centromeric cohesion have been established. Swi6, a conserved heterochromatin protein related to transcriptional silencing of centromeres, is required in *S. pombe* for Rad21 association with centromeres, but not with chromosome arms (Bernard *et al.*, 2001). Nonaka *et al.* (2002) showed that *S. pombe* Swi6 and its fly/mammalian homologue HP1 interact with Psc3, the *S. pombe* DSA1 homologue, and proposed a conserved role for Swi6/HP1 in centromere recruitment of mitotic cohesins. Concurring with this hypothesis, we found DSA1 along centromeric heterochromatin of *Drosophila* metaphase chromosomes, suggesting an important role for the heterochromatin in mitotic centromere cohesion during chromosome segregation in cultured fly cells. It is tempting to speculate that association of different cohesin complex subunits (or their differential modification) with heterochromatin proteins is involved in the distinction between arm and centromere cohesin complexes, and thus in the sequential release of chromatid arm and centromeric cohesion.

2.3. DSA1 and Drad21 cohesin subunits colocalize in all S2 cell cycle stages

Our results show that DSA1 is located in condensing prophase chromosomes in S2 cells, persisting at the centromeric regions until the metaphase/anaphase transition, similar to Drad21 localization (Warren *et al.*, 2000). RNAi and immunoprecipitation data suggested that Drad21 and DSA1 are part of a cohesin complex in cultured *Drosophila* cells (Vass *et al.*, 2003). To extend these biochemical results that indicate a DSA1/Drad21 interaction during the cell cycle, we double immunostained these proteins in S2 cells, and found complete colocalization in all cell cycle stages (Fig. 3). DSA1 and Drad21 colocalize on interphase cells (Fig. 3A),

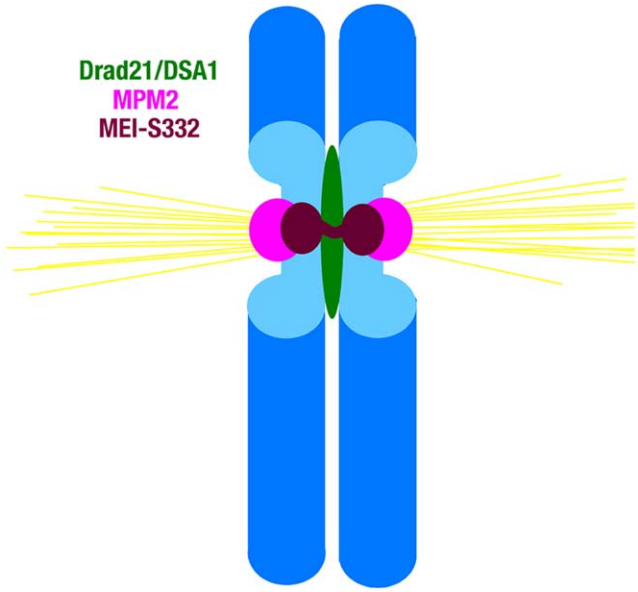

Fig. 4. **Schematic representation of the location of the DSA1 and Drad21 subunits of the cohesin complex in metaphase chromosomes**. In metaphase chromosomes DSA1 and Drad21 (green) colocalize and persist in the kinetochore pairing domain, as well as along the centromeric heterochromatin (light blue), maintaining centromere cohesion until the onset of anaphase. MPM2 phosphoepitope location is shown in pink, MEI-S332 is dark brown, microtubules are in yellow.

as well as prophase (Fig. 3B), prometaphase (Fig. 3C) and metaphase (Fig. 3D) chromosomes, and are found throughout the centromeric heterochromatin. This suggests their participation in centromere cohesion as subunits of a cohesin complex. The only dissimilarity in anaphase was in labeling of the spindle pole, where DSA1 appeared and Drad21 did not (Fig. 3E). Gregson *et al.* (2001) reported that a cohesin pool localizes to spindle poles in both metaphase and anaphase in mitotic HeLa cells, and showed that the mitotic spindle aster did not form in the absence of cohesin. Localization of the DSA1 signal to the spindle poles in anaphase (Fig. 3E) further supports these findings, potentially linking cohesin and the microtubule network in *Drosophila*.

Based on these results and on our previous findings, we propose that, in *Drosophila*, a cohesin complex composed of Drad21/DSA1/DSMC1/DSMC3 maintains sister chromatid arm cohesion during prophase. This complex persists at centromere heterochromatic regions, which are very large in *Drosophila*. As in vertebrates, this complex is resistant to removal by the prophase pathway. Protection of centromere cohesion is probably due to interaction of the cohesin complex with other centromeric proteins, for instance MEI-S332 (Fig. 4). Two *Drosophila* sequences encode SA/Scc3 homologues, DSA1 (this study) and DSA2, although only one Drad21/Scc1 coding sequence and no meiosis-specific REC8 coding sequences were identified in the *Drosophila* genome. In light of current understanding of sister chromatid cohesion in meiosis, it would be of interest to examine DSA1, DSA2 and Drad21 involvement in cohesion during insect meiosis.

3. Methods

3.1. Cell culture

Drosophila S2 cells were cultured at room temperature in Schneider's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 100 μg/ml penicillin and 100 μg/ml streptomycin.

3.2. Primary antibodies

Rabbit anti-DSA1 antibody was generated against recombinant DSA1 protein (Valdeolmillos *et al.*, 1998). Rabbit anti-Drad21 antibody was raised against a bacterially expressed carboxy-terminal Drad21 fragment (Warren *et al.*, 2000). Guinea pig anti-MEI-S332 antibody (kindly provided by Dr. T. Orr-Weaver) was raised against a full-length MEI-S332-GTS fusion protein (Tang *et al.*, 1998). Mouse monoclonal anti-MPM-2 antibody against mitotic phosphoproteins was from Upstate Biotechnology (Lake Placid, NY).

3.3. Immunofluorescence in S2 cells

Cells were resuspended in Schneider's medium (10^5 cells/ml); 100 μl were added to a drop of 0.3% BSA and cytocentrifuged onto an untreated slide (1,500 rpm, 5 min), then fixed in 3.7% formaldehyde, 0.5% Triton X-100 in PBS (10 min). Slides were washed in PBS-T (PBS with 0.05% Tween 20), blocked in 4% non-fat dry milk in PBS (1 h, room temperature), and incubated with primary antibodies diluted in PBS (overnight, 4°C). DSA1 was detected with rabbit anti-DSA1 antibody (1:100) and Drad21 with rabbit anti-Drad21 antibody (1:25). Centromeres were detected with mouse anti-MPM-2 mAb (1:100); MEI-S332 was detected with guinea pig anti-MEI-S332 antibody (1:5,000). Slides were washed in PBS-T as above, and incubated with secondary antibodies (30 min, room temperature).

A combination of Alexa 488-goat anti-rabbit IgG (1:400; Molecular Probes), with Cy3-goat anti-mouse IgG (1:400; Jackson) or Cy3-goat anti-guinea pig IgG (1:400, Jackson) were used for double immunolabeling. Slides were subsequently washed in PBS and mounted in Vectashield (Vector Laboratories) containing 1 μg/ml DAPI. In double immunolabeling experiments, primary antibodies were incubated simultaneously, except for DSA1/Drad21 co-labeling, for which slides were incubated with rabbit anti-DSA1 serum (1 h, room temperature), washed in PBS and incubated (overnight, 4°C) with FITC-conjugated goat Fab' fragment anti-rabbit IgG (1:100, Jackson). Slides were washed in PBS, incubated with rabbit anti-Drad21 serum (1 h), washed, and incubated with Cy3-goat anti-rabbit IgG (1:400, Jackson) for 30 min.

Observations were made using an Olympus BX-61 microscope equipped with epifluorescence optics. Images were captured with an Olympus DP50 digital camera and processed using Adobe Photoshop 6.0 software.

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